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Serum osteocalcin level is associated with glucose metabolism and atherosclerosis parameters in type 2 diabetes mellitus

Short title: Osteocalcin and Atherosclerosis

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Abstract

Introduction: Recent animal studies showed that osteocalcin action is related to not only bone metabolism but also to glucose metabolism and fat mass. We investigated the relationship between two bone formation markers, serum osteocalcin and bone specific alkaline phosphatase (BAP), and glucose metabolism, serum adiponectin, and the amount of fat mass as well as atherosclerosis parameters in men and postmenopausal women with type 2 diabetes.

Methods: A total of 179 men and 149 postmenopausal women were recruited consecutively, and radiographic and biochemical characteristics were collected. Brachial-ankle pulse wave velocity (baPWV) and intima-media thickness (IMT) were evaluated as the parameters of atherosclerosis.

Results: Multiple regression analysis adjusted for age, duration of diabetes, body mass index, and serum creatinine showed that osteocalcin negatively correlated with fasting plasma glucose and HbA_{1c} in both men and postmenopausal women ($p<0.05$) and with %Fat, baPWV, and IMT in men ($p<0.05$). Osteocalcin positively correlated with total adiponectin in postmenopausal women ($p<0.001$). After additional adjustments for systolic blood pressure, LDL-cholesterol, HDL-cholesterol, HbA_{1c}, and Brinkmann index, osteocalcin still significantly and negatively correlated with baPWV and IMT in men. In contrast, osteocalcin did not correlate with fasting C-peptide, and BAP did not correlate with any variable in either men or postmenopausal women.

Conclusions: Serum osteocalcin is associated with glucose and total adiponectin levels, fat mass, and atherosclerosis parameters in patients with type 2 diabetes, suggesting that osteocalcin is important for not only bone metabolism but also glucose and fat metabolism.

Introduction

Several studies have shown that osteoporosis is associated with cardiovascular disease and influences mortality (1-5). Although both diseases are traditionally viewed as separate entities that increase in prevalence with aging, accumulating evidence indicates that similar pathophysiological mechanisms lead to them. Arterial calcification, like osteogenesis, involves a complex interaction of various cells that produce matrix vesicles and subsequent mineralization. Previous studies have shown that bone-associated proteins, such as osteocalcin, Gla protein, osteopontin, osteoprotegerin, and receptor-activated nuclear factor-kappa B ligand (RANKL), were found in atherosclerotic arteries (6-8), suggesting that these proteins could be directly associated with vascular diseases.

Osteocalcin, one of the osteoblast-specific proteins, has several hormonal features and is secreted in the general circulation from osteoblastic cells (9,10). Recently, Lee *et al.* showed that osteocalcin functions as a hormone that regulates glucose metabolism and fat mass in genetically modified mouse (11). Osteocalcin-knockout mice display decreased β-cell proliferation, glucose intolerance, and insulin resistance. Moreover, Ferron *et al.* showed that osteocalcin administration regulated gene expression in β cells and adipocytes (including adiponectin expression), and affected the development of metabolic diseases, obesity, and type 2 diabetes in wild-type mice (12). Although these findings support the concept that bone metabolism and glucose/fat metabolism are associated with each other through the action of osteocalcin, little is known about whether or not serum osteocalcin level is associated with glucose, fat mass, adiponectin, or atherosclerosis parameters in humans.

In this study, to address this issue, we measured two bone formation markers, osteocalcin and bone specific alkaline phosphatase (BAP), as well as serum total and high molecular weight (HMW) adiponectin, body composition, abdominal fat area, and parameters of atherosclerosis, such as brachial-ankle pulse wave velocity

(baPWV) and ultrasonographically evaluated intima-media thickness (IMT); we measured these parameters in Japanese men and postmenopausal women with type 2 diabetes, and investigated their relationships to serum osteocalcin and BAP levels.

Methods

Subjects

The participants in this study were 179 men and 149 postmenopausal women with type 2 diabetes (age range, 50-83 and 50-84 years; mean 64.9 and 66.7, respectively). We consecutively recruited patients who visited Shimane University Hospital for education, evaluation, or treatment of diabetes. The baseline characteristics of the patients are shown in Table 1. All women had been without spontaneous menses for more than 1 year. Nobody had hepatic or renal dysfunction or nutritional derangements. The numbers of patients who had been taking insulin, sulfonylurea, metformin, and alpha-glucosidase inhibitors, respectively, were 37, 64, 23, and 19 men, and 39, 42, 32, and 14 women. Patients treated with thiazolidinedione were excluded from this study. Forty-seven men and 55 women had taken calcium antagonists; 38 men and 47 women had taken angiotensin converting enzyme inhibitors or angiotensin II receptor blockers for hypertension; 32 men and 54 women had taken HMG-CoA reductase inhibitors for dyslipidemia; and 35 men and 23 women had taken aspirin for atherosclerosis. Eighty-two men (45.8%) and 10 (6.7%) women were current smokers. The Brinkmann index was calculated by daily cigarette numbers multiplied by smoking years. All patients were free of drugs known to influence bone and calcium metabolism, such as vitamin D, bisphosphonate, or estrogen, up until the time of the study. This study was cross-sectional in design, approved by the ethical review board of our institution, and complied with the Helsinki Declaration. All patients agreed to participate in the study and provided informed consent.

Radiography

Fat mass was measured by

dual-energy X-ray absorptiometry (DXA) (QDR-4500; Hologic, Waltham, MA) using whole-body absorptiometry software and each value was expressed in kilograms. Percent fat mass was calculated by dividing each absolute value of body composition by total body mass. Percent trunk fat was calculated by dividing trunk fat mass by total fat mass. The coefficient of variation (precision) of measurements of fat mass was 2.0% (13).

Abdominal adipose tissue was measured using commercially available Computed Tomography (Toshiba medical systems, Tokyo, Japan), which determined adipose tissue area electronically by setting the attenuation values for the region of interest within a range of -150 and -50 Hounsfield units. Visceral fat area and subcutaneous fat area were determined separately with the use of a trace function, which manually defined the boundary between the visceral and subcutaneous fat with a cursor.

Biochemical measurements

After overnight fasting, serum was collected. Biochemical markers were measured by standard biochemical methods. Hemoglobin A_{1c} (HbA_{1c}) was determined by high performance liquid chromatography (HPLC). Serum C-peptide was measured by enzyme linked immunosorbent assay (ELISA). Osteocalcin and BAP were measured by radioimmunoassay (RIA) and Enzyme Immuno Assay (EIA), respectively, as previously described (14,15). Total adiponectin and HMW adiponectin were measured by ELISA kits (Otsuka Pharmaceuticals, Tokyo, Japan and Fujirebio, Tokyo, Japan, respectively), as indicated by the manufacturer. The coefficients of variation (CV) for total adiponectin and HMW adiponectin for each ELISA kit were 3.1 and 2.0%, respectively. Total cholesterol (TC), triglyceride (TG), and HDL-Cholesterol (C) were evaluated using an enzymatic method. LDL-C was calculated by Friedewald's formula [TC – (HDL-C +TG/5)] (16).

Arterial stiffness measurement

baPWV was measured using the VaSera VS-1000 (Fukuda Denshi, Tokyo, Japan), an automated recording device that calculates the time delay between two pulse waves recorded simultaneously, as previously described (17,18). Briefly, the patient was examined in the supine position, with a volume plethysmographic sensor in cuffs on both the brachia and ankles. After 15 minutes of rest, the subject's volume pulse form was detected, and time intervals between the wave front of the arm and that of the ankles were calculated. The distance between the arm and sampling points was calculated automatically. The CVs of measurements of L- and R-PWV were 1.37 and 1.31%, respectively. In the present study, the measurement of baPWV was performed on a different occasion from the blood collection so that the participant would be mentally relaxed.

Ultrasonographic measurement of carotid intima-media thickness

B-mode ultrasonographic imaging of the carotid artery was performed using HDI 5000 (Philips, Tokyo, Japan), a high-resolution, real-time ultrasonograph with a 7.5-MHz transducer, as previously described (17). All scans were performed by two trained sonographers who remained unaware of each other's data. Briefly, the scanning of extracranial carotid arteries in the neck was conducted bilaterally at longitudinal projections and at the transverse projection for measurement of IMT. Each carotid wall was explored to identify the thickest intima-medial sites. IMT was measured as the distance between the lumen-intima interface and the media-adventitia interface on the B-mode image. The CV of measurements of IMT was 3.55%. To quantify carotid artery wall thickness, we used the maximum of IMT in the present study.

Statistical analysis

Data were expressed as mean ± SD. Because serum total and HMW adiponectin showed a markedly skewed distribution, logarithmic (log) transformation of these values was carried out before

performing correlation and regression analysis. Simple and multiple regression analysis were performed using the statistical computer program Statview (Abacus Concepts, Berkeley, CA). $P < 0.05$ was considered to be significant.

Results

Baseline characteristics of patients and comparison of parameters between men and postmenopausal women

The baseline characteristics of the patients are shown in Table 1. We compared these parameters between men and postmenopausal women. Body height, body weight, visceral/subcutaneous fat ratio, serum creatinine, IMT, and Brinkmann index were significantly higher in the males than in the females ($p<0.001$). On the other hand, BMI, %Fat, subcutaneous fat area, LDL-C, HDL-C, total and HMW adiponectin, BAP, osteocalcin, and left baPWV were significantly lower in the males than in the females (different p values <0.05).

Correlations between serum levels of osteocalcin or BAP and adipose tissue, glucose metabolism, serum levels of adiponectin, and the parameters of atherosclerosis

Since our analysis showed that serum levels of osteocalcin, BAP, adiponectin, and the parameters of atherosclerosis (baPWV and IMT) were significantly affected by age, body stature, and renal function, multiple regression analyses adjusted for age, duration of diabetes, BMI, and serum creatinine were performed between serum levels of osteocalcin or BAP versus other variables (Table 2). In men, serum osteocalcin significantly and negatively correlated with %Fat, fasting plasma glucose (FPG), and HbA_{1c} ($p=0.0467$, $p=0.0013$, and $p=0.0318$, respectively), as well as right and left baPWV and IMT ($p=0.0032$, $p=0.0072$, and $p=0.0234$, respectively). In women, serum osteocalcin levels significantly and negatively correlated with FPG and HbA_{1c} ($p=0.0290$ and $p=0.0015$), and positively correlated with log (total adiponectin) ($p=0.0003$), but not with baPWV or IMT. In

contrast, serum BAP levels did not correlate with any parameter in either men or women.

The correlations between serum levels of osteocalcin or BAP versus the parameters of atherosclerosis

Next, to investigate whether serum osteocalcin levels in men were related to baPWV and IMT independent of other established cardiovascular risk factors, multiple regression analysis adjusted for age, duration of diabetes, BMI, serum creatinine, systolic blood pressure, LDL-C, HDL-C, HbA_{1C}, and Brinkmann index were performed between serum osteocalcin versus baPWV or IMT. In men, serum osteocalcin level was significantly and inversely correlated with right and left baPWV and IMT (r=-0.157, p=0.0149; r=-0.132, p=0.0332; r=-0.177, p=0.0263, respectively), even after being adjusted for those atherosclerosis-related parameters.

Discussion

In this study, serum osteocalcin level was significantly and negatively correlated with FPG and HbA_{1c} in both men and postmenopausal women with type 2 diabetes. Several studies indicated that hyperglycemia induces a low turnover of bone with osteoblast dysfunction and suppresses serum osteocalcin levels (19,20). Gerdhem *et al*. have shown that serum osteocalcin level, but not BAP, was lower in women with diabetes after correction for covariance of body weight and serum creatinine (20). Okazaki *et al*. have also shown that serum osteocalcin level was low before treatment and elevated after treatment of diabetes (21). Previous *in vitro* studies have shown that chronic hyperglycemia increases the activity and expression of alkaline phosphatase but decreases osteocalcin expression and cellular calcium uptake (22); this finding explains the discrepancy in serum levels of osteocalcin and BAP in clinical studies. Our findings are consistent with these observations and indicate that serum osteocalcin level, but not BAP, was specifically suppressed by hyperglycemia in diabetic patients.

Recent animal studies showed that

osteocalcin-knockout mice had glucose intolerance and insulin resistance (11), and osteocalcin administration improved these derangements by enhancing the expression of insulin genes and proliferation markers in pancreatic β cells (11,12). These data suggest not only that hyperglycemia suppresses osteocalcin expression in osteoblasts, but also serum osteocalcin secreted from osteoblasts into the circulation could modulate pancreatic β-cell function and improve glucose metabolism. However, we found that there was no correlation between serum osteocalcin level and fasting C-peptide, which is a surrogate marker for endogenous insulin secretion. This might be because patients in these studies have received several treatments that affect insulin secretion, including sulfonylureas and exogenous insulin. Therefore, we are unable to completely exclude the effects of these drugs when interpreting the association between serum osteocalcin level and insulin secretion.

Patients with osteoporosis are known to have an increased incidence of cardiovascular disease (1-5). Pennisi *et al.* have shown that low bone density and low bone formation markers, BAP and osteocalcin, were found in patients with severe atherosclerosis (23). In this study, we first investigated the statistical association of bone formation markers, osteocalcin and BAP, with atherosclerosis parameters. We found that serum osteocalcin level, but not BAP, was negatively associated with baPWV and IMT, independent of other atherosclerosis-related factors in diabetic men; this finding suggests that osteocalcin secreted from osteoblasts might be linked to atherosclerosis, although the amount of variability in the end point that was related to osteocalcin was generally small (r=-0.132 to -0.177). Recent evidence suggests that osteoblast-like cells are present in the vasculature and capable of calcifying vascular cells (24). Moreover, paracrine regulators of bone metabolism such as osteocalcin, matrix Gla protein (MGP), osteopontin, and bone morphogenetic protein (BMP) are also present in atherosclerotic arteries (25). Thus, the vascular microenvironment possesses mechanisms

similar to those in bone tissues to maintain mineral homeostasis. Both MGP and osteocalcin are known to be γ-Carboxyglutamic acid (Gla)-containing proteins. MGP is a secretory protein with widespread tissue expression, including in bone and vascular walls. MGP-knockout mice develop extensive calcification of arteries that rapidly becomes lethal, suggesting that MGP has an anti-mineralization role in the artery (26). In humans, osteocalcin is expressed parallel to MGP in both normal and atherosclerotic vessels (25), and is also detected in human carotid arteries from endarterectomy samples (8). Thus, the two Gla proteins, osteocalcin and MGP, could play a pivotal role in not only bone mineralization but also vascular wall calcification. However, at present, little is known about whether serum osteocalcin secreted from osteoblasts in bone or osteoblast-like cells in vessels actually could modulate atherosclerosis. Thus, further studies are needed to clarify the pathophysiological processes underlying the relationship between serum osteocalcin level and atherosclerosis parameters.

The present study showed that serum osteocalcin level was negatively correlated with %Fat in men with diabetes, and was positively correlated with serum total adiponectin level in postmenopausal women with diabetes. These findings suggest that osteocalcin is also associated with fat metabolism in persons with diabetes, although there were sex-related differences. Serum total and HMW adiponectins have been reported to be higher in postmenopausal women than in men (27,28), and our results also showed this sex-related adiponectin variability (Table 1). Ferron *et al.* have shown that the addition of osteocalcin to cultured white and brown adipocytes enhanced adiponectin expression in a dose dependent manner (12). These facts might partly explain the positive correlation between serum levels of osteocalcin and total adiponectin in postmenopausal women. On the other hand, we found no significant associations between osteocalcin and the parameters of atherosclerosis in postmenopausal women. These sex-related

differences might depend on background data such as higher serum adiponectin level and a higher percentage of non-smokers among women than men.

This study has certain limitations in addition to not excluding subjects who underwent diabetes treatment. First, the sample size was not large enough to make definite conclusions. Second, we analyzed only subjects who visited Shimane University Hospital, a tertiary center, for evaluation or treatment of diabetes mellitus and osteoporosis. Therefore, the patients enrolled in this study might have relatively severe states of the disorders and might not be representative of other Japanese men and postmenopausal women with the disorders.

In conclusion, we found that serum osteocalcin levels were associated with glucose and total adiponectin levels, fat mass, as well as atherosclerosis parameters in patients with diabetes. These findings support the recent notion that osteocalcin is important for not only bone metabolism but also glucose metabolism and fat mass (11,12).

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Table 1 Baseline characteristics of subjects

	Men			Women			p
number of subjects	179			149			
Age (years)	64.9	±	8.2	66.7	±	8.9	0.0583
Duration of diabetes (years)	12.0	±	9.1	12.0	±	10.0	0.9797
Body height (cm)	164.0	±	6.5	150.2	±	5.7	<0.0001
Body weight (kg)	61.0	±	9.6	54.4	±	10.4	<0.0001
BMI (kg/m^2)	22.6	±	2.9	24.1	±	4.4	0.0003
Systolic blood pressure (mmHg)	128	±	16	130	±	19	0.3213
Diastolic blood pressure (mmHg)	76	±	11	74	±	11	0.0976
%Fat (%)	19.5	±	4.4	29.5	±	6.7	<0.0001
%Trunk fat (%)	49.9	±	5.9	50.9	±	6.4	0.2389
Visceral fat area (cm^2)	113.0	±	64.1	105.9	±	64.2	0.3823
Subcutaneous fat area (cm^2)	103.2	±	49.1	181.6	±	95.8	<0.0001
Visceral/subcutaneous fat ratio	1.17	±	0.62	0.68	±	0.74	<0.0001
FPG (mg/dl)	173	±	57	164	±	62	0.1825
HbA1c (%)	9.1	±	2.4	8.9	±	2.5	0.5162
Fasting C-peptide (ng/ml)	1.6	±	0.8	1.6	±	0.8	0.7358
LDL-C (mg/dl)	108	±	34	119	±	39	0.0074
HDL-C (mg/dl)	52	±	16	58	±	17	0.0008
Serum creatinine (mg/dl)	0.78	±	0.15	0.62	±	0.15	<0.0001
Total adionectin (μg/ml)	6.13	±	3.64	8.88	±	5.84	<0.0001
HMW adiponectin (μg/ml)	5.90	±	4.82	9.18	±	7.38	<0.0001
BAP (IU/l)	26.1	±	8.9	32.6	±	12.4	<0.0001
Osteocalcin (ng/ml)	5.2	±	2.3	7.2	±	3.0	<0.0001
Right-baPWV (m/s)	15.0	±	2.6	15.5	±	2.6	0.1037
Left-baPWV (m/s)	14.7	±	2.2	15.4	±	2.7	0.0145
Intima-media thickness (mm)	2.4	±	1.3	2.0	±	0.9	0.0009
Brinkmann index	609	±	653	21	±	114	<0.0001

BMI, body mass index; HMW, high molecular weight; FPG, fasting plasma glucose; HbA_{1c}, hemoglobin A_{1c}; CPR, C-peptide; IMT, intima-media thickness; baPWV, branchial-ankle pulse wave verocity; BAP, bone specific alkaline phosphatase

Table 2 The correlations between serum levels of osteocalcin or BAP versus adipose tissue, glucose metabolism, serum levels of adiponectin, or the parameters of atherosclerosis.

	osteocalcin				BAP			
	Men		Women		Men		Women	
	r	p	r	p	r	p	r	p
%Fat	-0.133	0.0467	-0.003	0.9592	-0.084	0.2146	0.086	0.1220
%Trunk fat	-0.113	0.1282	-0.113	0.2453	-0.093	0.2212	0.001	0.9906
Visceral fat area	0.038	0.6171	-0.049	0.4856	-0.087	0.2486	0.001	0.9934
Subcutaneous fat area	0.040	0.5538	0.027	0.6277	-0.026	0.6929	0.009	0.8754
Visceral/subcutaneous fat ratio	-0.048	0.5883	0.118	0.2383	-0.042	0.6395	0.145	0.1513
Fasting plasma glucose	-0.242	0.0013	-0.190	0.0290	0.070	0.3479	-0.162	0.0674
HbA_{1c}	-0.164	0.0318	-0.274	0.0015	0.097	0.1936	-0.111	0.2136
Fasting C-peptide	-0.064	0.4038	0.039	0.6146	-0.035	0.6400	0.014	0.8535
Log(Total adionectin)	-0.023	0.7666	0.301	0.0003	0.104	0.1700	0.031	0.7281
Log(HMW adiponectin)	-0.002	0.9844	0.136	0.1059	0.148	0.0731	0.046	0.5927
Right-baPWV	-0.204	0.0032	-0.114	0.0914	-0.045	0.5169	-0.061	0.3778
Left-baPWV	-0.183	0.0072	-0.055	0.4176	-0.051	0.4495	-0.043	0.5313
Intima-media thickness	-0.181	0.0234	0.022	0.8029	0.015	0.8463	0.149	0.1010

Multiple regression analysis was performed between osteocalcin or BAP versus adipose tissue, glucose metabolism, serum adiponectin, or the parameters of atherosclerosis adjusted for age, duration of diabetes, body mass index, and serum creatinine.

BAP, bone specific alkaline phosphatase; HbA_{1c}, hemoglobin A_{1c}; Log, logarithm; baPWV, branchial-ankle pulse wave velocity